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DNA as an enzyme: The effect of imidazole derivatives as cofactors and metal ions as activators or inhibitors

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Abstract

Objective: a highly sensitive spectrofluorometric method using dichlorofluorescin (LDCF) was employed to study the rate of electron transfer reaction in presence of DNA and some imidazole derivatives.
Results: in our experiments, DNA possessed a unique enzyme like catalytic function in oxidative conversion of nonfluorescent LDCF to fluorescent dichlorofluorescein (DCF). The rate enhancement was associated with the turn over constant: $k_p = 10\ s^{-1}$ for DNA and cinnamoyl imidazole as a cofactor. A biphasic saturation curve was observed when the reaction velocities were measured at fixed concentrations of DNA and variable amounts of carnosine. Each of the biphasic trends gave the Scatchard values of $V_{m1}/K_{m1} = 3.1 \times 10^{-5}$ and $V_{m2}/K_{m2} = 5.1 \times 10^{-6}$ with $K_{m1} = 2.7 \times 10^{-5}$ M and $K_{m2} = 4.2 \times 10^{-4}$ M for carnosine. Although Ni (II) and Pb (II) induced inhibition in the rate of electron transfer reaction in presence of DNA and cinnamoyl imidazole or carnosine, metal ions such as Mg (II), Cd (II), Zn (II) and Fe (II) caused activation of DNA. The rates of the reactions showed strong dependency on electronegativity and conductivity of metal ions, namely the increase in activity of DNA in presence of each metal ion correlated inversely with the electronegativities of the metal and was also related directly to the conductivities of individual metal. These effects were observed both in activation and also inhibition of DNA reaction. Imidazole compounds, *e.g.,* Histidine, N-*trans* cinnamoyl imidazole and imidazole along with Cd (II) produced further rate enhancement. The increase was several times greater with N-*trans* cinnamoyl imidazole.
Conclusions: this effect could provide additional evidence for the importance of an intermediary cofactor that could facilitate the transfer of the electron from the reaction site to the DNA conductive chord. This was most guaranteed by the conjugated system provided by a compound such as N-*trans* cinnamoyl imidazole.

Keywords: DNA catalysis; imidazole derivatives; activation and inhibition; metal ions; fluorescence

1. Introduction

The activation *via* oxidation of diacetyl dichlorofluorescin (LDADCF) to a nonfluorescent dichlorofluorescin (LDCF) and to fluorescent dichlorofluorescein (DCF) has been used to detect ultra-micro quantities of hydroperoxides, *i.e.*, lipid hydroperoxides as well as H_2O_2 detection in cellular processes [1,2] and in enzyme systems such as in glucose oxidase [3,4].

A method using LDCF was also employed to detect and evaluate the oxidative burst in neutrophiles [5]. Topographic distribution of oxidative stress, temporal alteration in microcirculatory units of isolated perfused liver could also be studied by such method. The reaction pathway in oxidative conversion of LDADCF to LDCF and DCF could be delineated as follows (Fig. 1).

Hematin (a heme derivative) is used in this conversion to accelerate the effect of hydrogen peroxide. It is thought that a complex is formed between hematin and hydrogen peroxide that could dissociate into a ferryl-oxo compound and a hydroxyl radical, which assist oxidation of LDCF to DCF [2,6].

It is suggested in the literature that a free radical or superoxide radical may be involved in hydroxylation reaction of guanine in DNA. On the other hand, the role of carnosine (β-alanyl-L-histidine), an endogenous dipeptide, is known to be as a free radical scavenger and an antioxidant [7,8] and was reported to have an important part in con-

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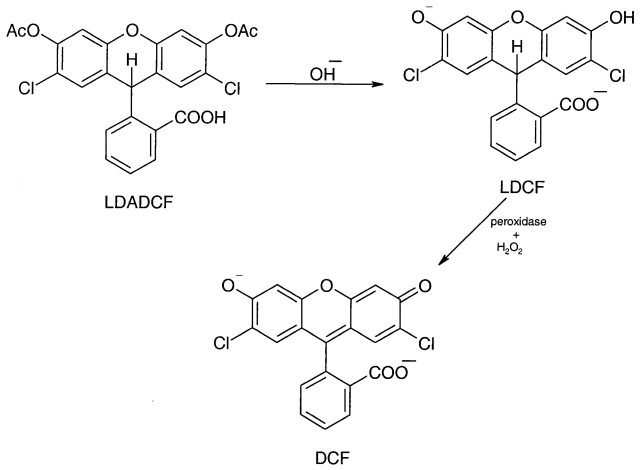

Fig. 1. Reaction scheme of activation and oxidation of diacetyl-dichlorofluorescein (LDADCF) to dichlorofluorescein (DCF).

junction with metals such as Ni (II) in C8 hydroxylation of guanine [9]. Datta *et al.* have employed superoxide dismutase to detect the presence of superoxide in C8 hydroxylation reaction with no succesful results [9].

Our initial study was mainly aimed at finding weather a free radical and/or electron transfer is involved in such hydroxylation by using similar components of the reaction, *i.e.*, Ni-Carnosine and guanine, in presence of another electron transfer process associated with the conversion of LDCF to DCF. No interference with the rate of the reaction and no results were obtained with such reaction components.

Based on the reports on electrical potentials in double helical structure of DNA [10–15] and other reports indicating that any aberration in the structure of DNA results in obstruction of electrical flow [16], we undertook the study of the electron transfer reaction in presence of DNA to observe if such properties of DNA could interfere with the electron transfer reaction rate. Our preliminary results [1], showed a rate enhancement in conversion reaction of LDCF to DCF in presence of DNA and carnosine. Ni (II) could act as an inhibitor in this reaction. In continuation of the study, effect of different divalent cations and some other imidazole derivatives were investigated.

2. Material and Methods

2.1. Materials

2′, 7′- dichlorofluorescin diacetate (LDADCF), carnosine and other imidazole derivatives were from Sigma Chemical Co. Hydrogen peroxide, Hematin and the chloride salts of Mg^{2+}, Zn^{2+}, Ni^{2+}, Fe^{2+} and Cd^{2+} as well as nitrate of Pb^{2+} were from Merck Chem. Co. High molecular weight DNA was extracted and purified from calf-thymus as explained elseware [17].

2.2. Methods

2.2.1. Dichlorofluorescin solution

Stock solution of LDADCF (1 mM) was made in ethanol and stored in the dark. LDADCF is stable for months under this condition. Activation of LDADCF to LDCF for assay required dilution of 1:4 V/V of ethanol and 0.01 N of sodium hydroxide. The mixture was allowed to stand at room temperature for 30 min. LDCF has a high rate of autoxidation, so it required being prepared freshly each day.

2.2.2. Hematin solution

The hematin solution (0.01 mg/mL) was prepared by dissolving 1 mg of hematin in 0.5 mL of 0.2 N NaOH and then diluted to 100 mL with 50 mL 0.05 mol/L Tris-HCl buffer. This solution was made freshly each day.

2.2.3. Sample preparation

14 mL of hematin solution was made up to 100 mL with 0.05 mol/L tris-HCl buffer in various pH and boiled for 15 min, while purging with nitrogen. This solution was cooled on ice. To prepare the medium for the reactions, a 2.7 mL volume of this solution was mixed with 200 μL of activated dichlorofluorescin (1.1×10^{-5} M) and 100 μL hydrogen peroxide (1.1×10^{-5} M). Ultimately DNA, the imidazole containing compounds and metal ions were added. Then the sample mixtures were incubated under a nitrogen atmosphere at 50°C for 45 min. The reaction was cooled to room temperature and the relative fluorescence was determined using a spectrofluorometer (Shimadzu, Model RF-5000) with a 4-mL fluorescence cell. The excitation and emission wavelengths were 496 and 521.6 nm, respectively. Both of excitation and emission slits were in 5-nm bandwidth. For the determination of optimal pH, all the required amounts of reagents were made in tris buffer adjusted to the desired pH between 6.8 to 9 with 0.2 pH units intervals. The DNA activity was measured at each pH. The metal ion concentrations in samples ranged from 2×10^{-4} to 12×10^{-4} M. The imidazole derivatives were used in saturating concentrations of 1.8×10^{-4} M.

2.2.4. DNA activity measurement

DNA activity was measured from the following equation:

$$\text{DNA activity} = \Delta \text{ emission}/\epsilon \times \text{time}$$

where Δ emission is the change of fluorescence emission as a function of time. The ϵ is the extinction coefficient of DCF, which was obtained by measuring the molar absorptivity of product of the reaction at the infinite time.

3. Results

3.1. The effect of DNA and carnosine

Initially the pH activity profile was determined using all components of the reaction in tris buffer 0.05 mol/L pH 6.8

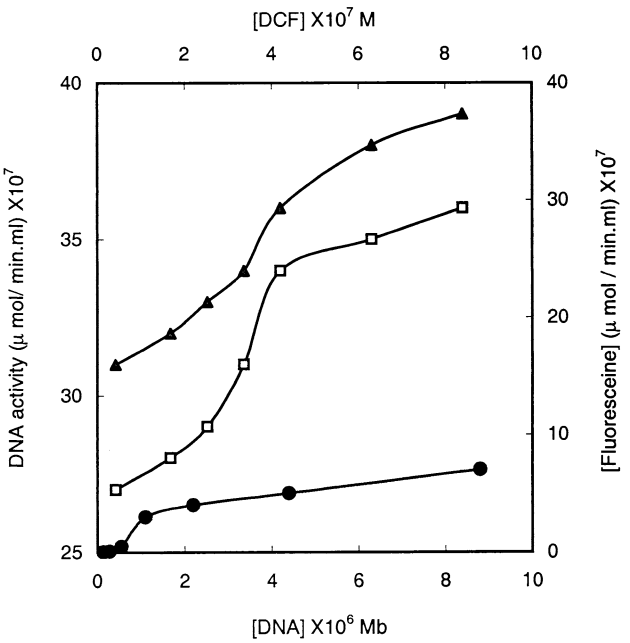

Fig. 2. DNA activity as a function of DNA concentration in the absence (square) and presence of carnosine (triangle) as shown in the first axes, [carnosine] = 1×10^{-4} M. The reaction without DNA and carnosine as blank (circle) is shown on the second axes.

to 9. The optimum pH was found to be 8.4 for such reaction. This pH was used throughout all activity measurements (data not shown).

The rate of the conversion of LDCF to DCF in the absence of DNA and carnosine at optimal pH 8.4 and the enhancement in the rate by DNA in presence and/or absence of carnosine is shown in Fig. 2. The Scatchard plot for DNA reaction (Fig. 3B) is plotted based on the biphasic trend of rate enhancement with increasing concentrations of carnosine in the presence of fixed concentrations of DNA (Fig. 3A). Scatchard plot, shows a two well defined linear segments (treated separately according to the method described at Ref. [18]) the K_m and V_m values are obtained for each segment. K_{m1} and K_{m2} are found to be equals to 2.7×10^{-5} M and 4.2×10^{-4} M respectively. Also the values of V_{m1} and V_{m2} are found to be equal to 7.5×10^{-10} M/h and 2.1×10^{-9} M/h respectively. Therefore, it may be stated that the acceleration of the rate of LDCF to DCF could be *via* a catalytic route induced by DNA in which DNA portrays an enzyme like behavior with carnosine as a coenzyme. The mechanism of action of carnosine as a prooxidant has been described variably in different reports [7–9,19].

To observe if any side reactions such as binding of DNA and/or carnosine to LDCF or DCF were not affecting the rate enhancement, we incubated LDCF with DNA and carnosine without other components of the reaction, *i.e.*, hematin and hydrogen peroxide and examined the change in fluorescence intensity periodically from initial zero time to several hours. No detectable changes were observed. The possibility of direct interaction of DCF with DNA or carnosine was also excluded since when excess DNA or carnosine was added to the product of the reaction (DCF) no detectable changes were observed (Fig. 2).

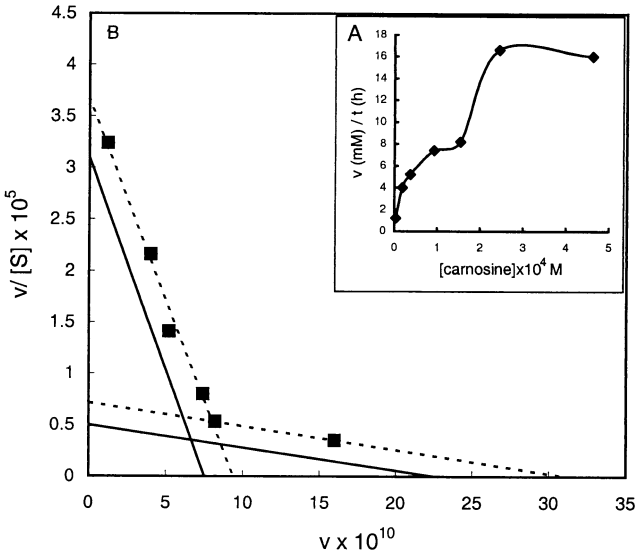

Fig. 3. A: DNA biphasic saturation curve with carnosine increasing concentrations, [DNA] = 8.4×10^{-6} Mb. Each point is representative of a change in fluorescent intensity vs. time (minutes). *B*: Scatchard plot was obtained from the data of Fig 3A.

3.2. The effect of metal ions

Fig. 4 depicts the rate enhancing and inhibiting effect of various metal ions. As it is shown in the plots, Cd (II)

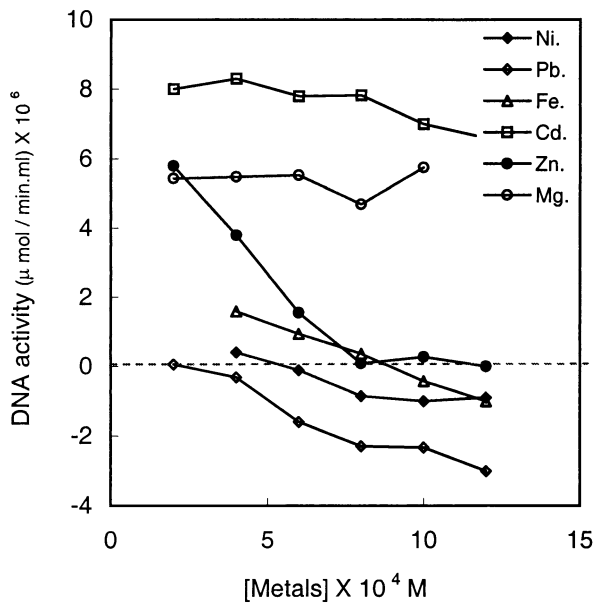

Fig. 4. Relative activity of DNA ([DNA] = 8.4×10^{-6} Mb) in DCF reaction with increasing concentrations of different metal ion: Cd (II), Mg (II), Zn (II), Fe (II), Ni (II) and Pb (II). Each point represents the activity of DNA at a specific concentration of metal ion. The DNA activities without metal ions were subtracted from each point.

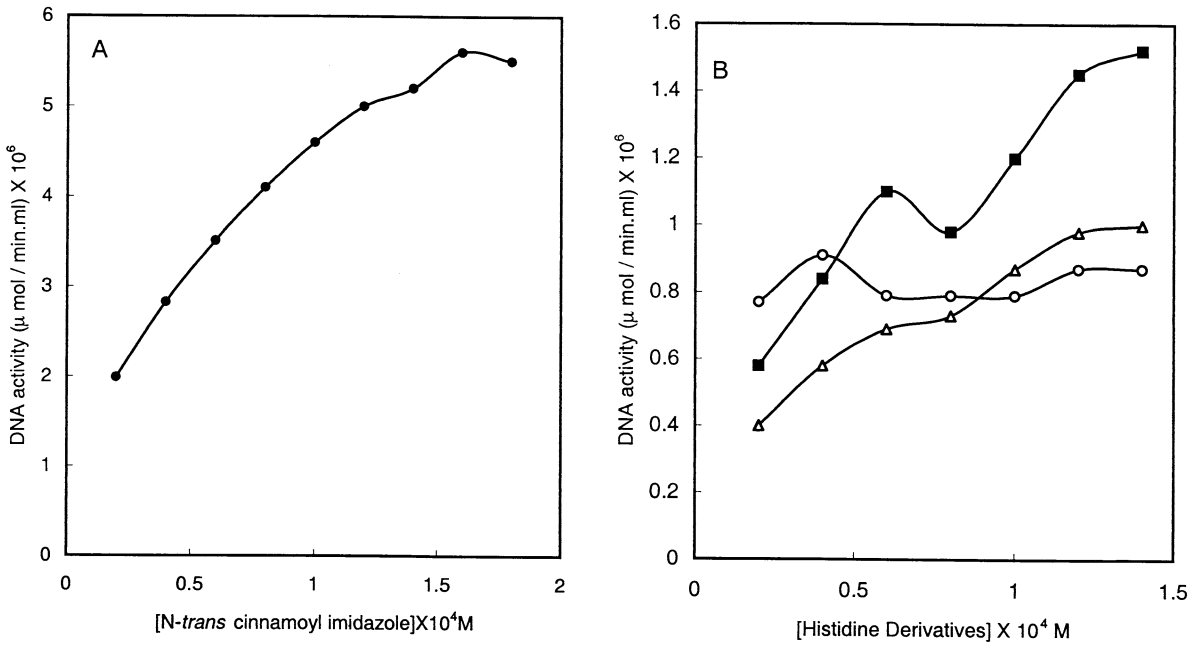

Fig. 5. The effect of increasing concentration of imidazole derivatives in DNA catalytic activity; $[CdCl_2] = 4 \times 10^{-4}$ M and [DNA] = 8.4×10^{-6} Mb; A: N-*trans* cinnamoyl imidazole; B: carnosine (triangle), histidine (circle) and imidazole (square).

enhanced the rate of the reaction to a maximum of 8 μmol/min.ml of DNA activity, which was eight times higher, compared to the reaction with no metal ions (the zero base line). This effect was solely ascribed to activation of DNA catalytic reaction by metals. The possible interference of other reactions was eliminated by using two blanks, one with DNA in the absence of metal ions and the other without DNA and in the presence of metal ions. The enhancement in catalytic activity of DNA could also be observed with metal ions such as Mg (II) and Zn (II).

3.3. The effect of imidazole compounds

Imidazole compounds such as N-*trans* cinnamoyl imidazole, imidazole, histidine and carnosine used in DNA reaction produce different degrees of DNA activation. Since we obtained the highest activity of DNA with Cd (II), this metal ion was employed in this study. Our results showed that N-*trans* cinnamoyl imidazole was most effective in catalyzing DNA reaction compared to others (Fig. 5A). Imidazole, histidine and carnosine were less effective. The order of their effectiveness was shown to be N-*trans* cinnamoyl imidazole> Imidazole> histidine ≥ carnosine (Fig. 5A and B).

4. Discussion

The oxidative conversion of LDCF to DCF (the fluorescent product) is performed in the presence of hydrogen peroxide, as an oxidant. Hematin, the other component of the reaction mixture was suggested to form a complex with hydrogen peroxide. This complex was dissociated into a ferryl-oxo and a hydroxyl radical, where both could accelerate DCF formation from LDCF [2,6]. Most heme proteins such as hematin facilitate this process. The optimal concentrations of these components in the reaction were determined and the effect of various factors *e.g.*, the pH, DNA and carnosine concentration were studied. Although the results obtained indicated that DNA or carnosine could enhance the rate of the reaction at optimal pH of 8.4, but the reaction was more effectively catalyzed by both factors. Considering the results obtained from carnosine enhancing effect on DNA reaction, it could be assumed that the electron transfer from the LDCF conversion to DCF could be mediated by carnosine to the DNA structure which has been found to contain electrical flow [11]. This assumption is more strengthened when we used other imidazole derivatives and metal ions.

We initially used Ni (II) to observe its role in the rate of the reaction and an inhibition was observed. Other metal ions were used to investigate their role in this catalytic reaction. As shown in Figure 4 Pb (II) could also act as an inhibitor, although Cd (II), Mg (II), Zn (II) and Fe (II) could enhance the rate of the reaction. We assumed that the enhancing effect of metals could be correlated to their electronic structure. The common characteristics of these metals are their filled orbital ending with s^2. It could be rationalized that metal ions bind to phosphate oxygen could compensate their charges and mediate more effectively the transfer of electrons from the reaction pathway to the DNA structure. Other metals in our studies such as Pb (II) and Ni

(II) were found to inhibit the reaction. Based on similar rationalization, these metals contain unfilled inner orbital and therefore could act as electron sinks when bound to DNA. Fe (II) in our studies was found to activate the reaction in low metal ion concentrations, although caused an inhibition in higher concentrations. The activation of DNA by Zn (II) in lower metal concentration and its subsequent decrease due to increasing concentration of Zn (II) could be assigned to a second reaction, possibly a ligand formation between the metal ion and DNA structure forming a square planar structure that could interfere with the original role of Zn (II) as an electron transporter. It is noteworthy to mention that in all instances an inverse correlation was found between the activity of DNA in presence of metal ions and their electronegativities. Another relationship was obtained between the conductivity of metal ions with the rates of the reaction. This could support the hypothesis that the flow of electrons through the DNA structure is enhanced with metal ions that could act as better conductors. It may be envisaged that the metal ions could act as a bridge in transferring the electrons received from the course of reaction *via* the imidazole derivatives to the DNA electron flow. An exception was observed with Cd (II), which showed higher activation of DNA, which was not online with other metals. In the first instance we assumed that it might be due to a different catalytic mechanism other than its capacity to transfer the electrons. In further studies we found that Cd (II) has different atomic parameters *i.e.*, higher atomic radius and lower density; that was relatively different than the metals in the same series. Therefore it could be suggested that the higher enhancement caused by Cd (II) may be due to some ionic characteristics such as softness and relative electropositivity of metal ion compared to other members of the series [20,21].

Comparing the imidazole derivatives used in this study (Fig. 4), carnosine, imidazole, histidine and N-*trans* cinnamoyl imidazole, could enhance the DNA activity, although N-*trans* cinnamoyl imidazole showed the highest effect. Considering the conjugated structure of cinnamoyl imidazole in which the aromatic ring is placed on one side and an imidazole group on the other side, a system is formed in which an electron that may be absorbed by the aromatic ring could be conducted through the conjugated system and transferred by imidazole group to the metal ion and consequently to DNA structure.

It could be assumed that the overall role of imidazole derivatives was to accelerate the rate of the reaction through mediating the transfer of electrons from the center of the reaction *via* the metal ion to the conductive chord of DNA. Therefore a process of catalysis can be evidenced. The metal ions, which inhibit the reaction, are those with carcinogenic potentials [22–25]. This points to the possibility that the restriction in the transport of electron through DNA could be related to the carcinogenic effect of these metals. Other reports indicated that restriction in the flow of electron when two or more guanine residues are placed adjacent to each other could act as an electron pool, leading to C8-hydroxylation and DNA aberration [25,26].

A more detailed study of DNA catalysis, activation and inhibition is called for to clarify further both the nature of such phenomena and also its impact on the biologic systems.

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